ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1983, p. 10–14
0066-4804/83/070010-05$02.00/0

Vol. 24, No. 1

Phosphorylation of Acyclovir In Vitro in Activated Burkitt Somatic Cell Hybrids

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Received 10 December 1982/Accepted 14 April 1983

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] (ACV), a potent antiviral compound, was phosphorylated to the same extent by extracts from untreated and iododeoxyuridine-treated Epstein-Barr virus-containing latent D98/HR-1 somatic hybrid cells. ATP was the preferred phosphate donor over other nucleoside triphosphates. The cytosol extract from D98/HR-1 cells effected optimum phosphorylation of thymidine at pH 8.0, whereas ACV was phosphorylated equally well over a wide pH range. Electrophoretic analysis of thymidine kinase-, deoxycytidine kinase-, and ACV-phosphorylating activities from both untreated and iododeoxyuridine-treated cell extracts displayed identical properties. A small part (5 to 10%) of the loaded ACV-phosphorylating activity seemed to migrate with the deoxycytidine kinase activity from cytosol. dTTP and dCTP, at relatively high concentrations, partially inhibited ACV-phosphorylating activity. The results suggest that Epstein-Barr virus does not code for its own thymidine kinase and that phosphorylation of ACV in Epstein-Barr virus-producing cells is carried out by multiple or as yet unidentified ATP-dependent nonspecific cellular phosphotransferases.

The acyclic nucleoside analog acyclovir [9-(2-hydroxyethoxymethyl)guanine] (ACV) is currently considered as one of the drugs for the topical treatment of certain herpes simplex virus (HSV) infections, such as primary genital herpes and generalized cutaneous herpes in immunocompromised patients (10). Biochemical and genetic studies of its mechanism of action in HSV systems have shown that the compound is converted to its triphosphate form; the first step of phosphorylation is carried out by virus-specified thymidine (TdR) kinase (4, 10). The monophosphate thus formed is subsequently converted to triphosphate by host enzymes (17). The triphosphate form specifically inhibits the DNA polymerase of HSV by competing with dGTP (10). Cells infected with TdR kinase-negative virus were unable to phosphorylate ACV to a great extent, and hence the drug was ineffective.

Reports from this laboratory (6, 7) show that ACV is effective in inhibiting Epstein-Barr virus (EBV) replication. However, in contrast to HSV, the amount of phosphorylated drug formed in vivo in EBV-infected cells is minimal and identical in both infected and noninfected cells (5). These observations led us to analyze the process of ACV phosphorylation in vitro in a latent EBV DNA-containing cell line (D98/HR-1, 10a).

Treatment of D98/HR-1 somatic cell hybrids with iododeoxyuridine (IUdR) gives rise to the induction of EBV-associated DNA polymerase with a subsequent induction of viral DNA synthesis and the production of EBV particles (16). The induction of viral DNA synthesis in IUdR-treated cells can be efficiently inhibited by ACV (15). This system was ideally suited for our purpose inasmuch as we could follow the induction of EBV-associated DNA polymerase activity as an internal marker while analyzing the extent of ACV phosphorylation and TdR kinase activity. This exercise was necessary in view of the conflicting reports in favor of both existence and nonexistence of EBV-associated TdR kinase (3, 19, 20). Moreover, in vaccinia virus and herpesvirus saimiri systems, ACV is not at all effective despite the presence of virus-specified TdR kinase (10, 11). These findings raised the question of whether the presence of virus-specified TdR kinase is essential for ACV to be effective. The studies reported in this paper are directed at answering this question.

MATERIALS AND METHODS

Cells. Burkitt hybrid cells, D98/HR-1, clone 1, selected in hypoxanthine-aminopterin-thymidine-containing medium were a gift of Ronald Glaser, Ohio State University, Columbus.

Chemicals. [^{14}C]ACV (54 mCi/μmol) and unlabeled ACV were gifts of G. Elion, Burroughs Wellcome Co., Research Triangle Park, N.C. Nucleosides and their triphosphates were obtained from P-L Biochemicals, Milwaukee, Wis. ATP was purchased from Sigma Chemical Co., St. Louis, Mo. Tetrahydrouridine was provided by Y.-C. Cheng, Cancer Research Center, University of North Carolina, Chapel Hill.

Treatment of cells with IUdR. Cells were seeded in minimal essential medium and treated with IUdR for 3 days as described elsewhere (15).

Preparation of phosphorylating activity. Mock-treated and IUdR-treated cells were processed as follows. Cells were suspended in a buffer containing 10 mM KCl, 0.0015 M $MgCl_2$, 10 mM Tris (pH 7.4), and 0.25 M sucrose and kept suspended for 20 min at 0°C. Phenylmethylsulfonyl fluoride was then added to a final concentration of 1 mM, and the cells were disrupted with a Dounce homogenizer. The suspension was centrifuged at 800 × *g* for 5 min. The pellet formed was used as the source of nuclei. KCl was added to the supernatant fluid to a final concentration of 0.15 M, and ATP and β-mercaptoethanol were added to final concentrations of 10 and 5 mM, respectively. The supernatant fluid was centrifuged at 105,000 × *g* for 2 h. The clear supernatant fluid formed was dialyzed for 8 to 12 h against a dialysis buffer (10 mM Tris [pH 7.4], 0.15 M KCl, 10 mM β-mercaptoethanol, 10 mM ATP, 10 mM $MgCl_2$, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride). The dialyzed fraction was clarified by centrifugation and was used as the cytosol extract. Total cell extract was prepared by suspending the cells in the same dialyzing buffer and then sonicating them. The rest of the procedure was the same as for the cytosol preparation. The washed nuclear pellet used for DNA polymerase assays was processed as described before (8).

Enzyme assays. Assays for TdR kinase were carried out as follows. The reaction volume of 100 μl contained 100 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP, 3 mM β-mercaptoethanol, 2 mM NaF, 100 μM [^{3}H]TdR (10 cpm/pmol), and the required amount of enzyme. The reactions were carried out at 37°C for 30 min. Samples (50 μl each) of the reaction mixture were removed and applied to DE-81 paper disks. The papers were washed three times with 0.01 M ammonium acetate, followed by two rinses with water and with 95% alcohol. Filters were dried, and radioactivity was determined in toluene-based solvent.

For deoxycytidine (CdR) kinase assay the methods were the same except that [^{3}H]CdR was used as the substrate, and the reaction mixture contained 0.2 mM tetrahydrouridine.

The reaction mixture for assay of ACV phosphorylation was exactly the same as for the TdR kinase assay except that 500 μM [^{14}C]ACV (54 cpm/pmol) was used as the acceptor nucleoside. Assays were carried out for 120 min unless otherwise stated. For determination of radioactivity, the DE-81 papers were rinsed three times with 0.001 M ammonium acetate, three times with 70% alcohol containing 1 mM guanosine and 0.001 M ammonium acetate, and three times each by washing under pressure filtration with water and with 70% alcohol.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was essentially the same as described elsewhere (13) except that ammonium persulfate was used instead of riboflavin for gel polymerization. Samples (50 μl each) in 20% glycerol–0.001% bromophenol blue were applied to the gel. After PAGE, the gels were sliced and assayed by dipping each slide in 100 μl of reaction mixture. The incubations were for 3 at 37°C for ACV phosphorylation and 1 for TdR and CdR phosphorylation.

RESULTS

ACV- and TdR-phosphorylating activities in IUdR-treated D98/HR-1 cytosol extracts. ACV was phosphorylated equally by both untreated and IUdR-treated cytosol extracts (Fig. 1). The reaction was linear over a period of 120 min. However, the rate of ACV phosphorylation was 100 to 150 times lower than the rate of TdR phosphorylation. The phosphorylation was mostly ATP dependent (Table 1) in both mock-treated and IUdR-treated cytosol extracts.

To ascertain whether TdR kinase present in the cytosol extract plays any role in the phosphorylation, ACV and TdR kinase assays were carried out at different pHs. TdR was phosphorylated maximally at pH 8.0 and had a sharp pH optimum (Fig. 2). In contrast, phosphorylation of ACV had very broad pH optima in both IUdR-treated and untreated extracts.

PAGE analysis of TdR kinase-, CdR kinase-, and ACV-phosphorylating activity. For further insight into the different deoxynucleoside kinase activities in IUdR-treated D98/HR-1 cells, the whole-cell extracts from IUdR-treated and un-

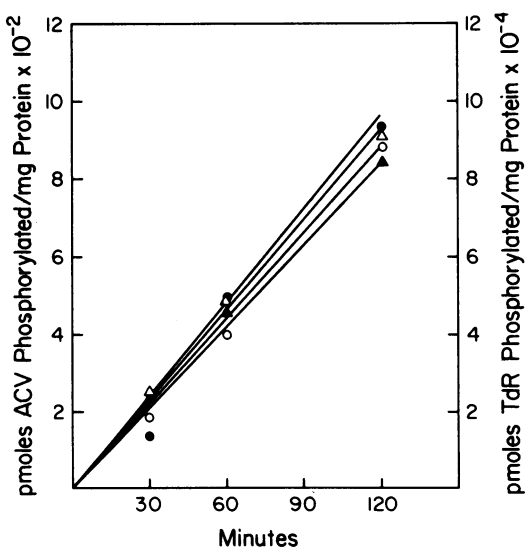

FIG. 1. Kinetics of phosphorylation of ACV and TdR by the cytosol extracts from IUdR-treated and untreated D98/HR-1 cells. ACV phosphorylation with untreated (△) and IUdR-treated (▲) cellular cytosol extracts and TdR phosphorylation with untreated (○) and IUdR-treated (●) cellular cytosol extracts were carried out as described in the text.

TABLE 1. Phosphate donor specificity for ACV phosphorylation[a]

Phosphate donor	pmol of ACV phosphorylated per mg of protein	
	Untreated D98/HR-1 cytosol	IUdR-treated D98/HR-1 cytosol
None		
AMP	180	169
ADP		
ATP	1,505	1,449
GTP	449	637
CTP	498	409
UTP	403	497
TTP	13	17

[a] Assays were carried out with cytosol extracts.

treated cells were analyzed by PAGE (Fig. 3). The results indicated that in both treated (Fig. 3A) and untreated (Fig. 3B) extracts there were two TdR kinase isozymes with different electrophoretic mobilities. The activity with slower electrophoretic mobility (0.15) is cytosol kinase, whereas mitochondrial kinase moved with an electrophoretic mobility of 0.6. The identity of

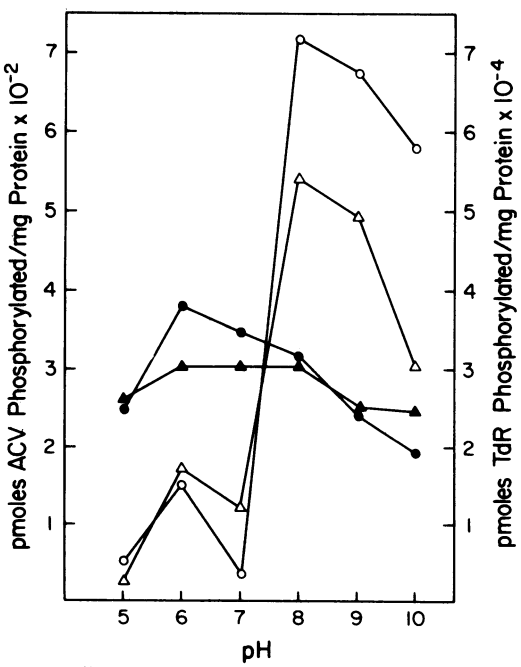

FIG. 2. Determination of optimum pH for phosphorylation of ACV and TdR. ACV phosphorylation with untreated (●) and IUdR-treated (▲) cellular cytosol extracts and TdR phosphorylation with untreated (○) and IUdR-treated (△) cellular cytosol extracts were carried out at different pHs.

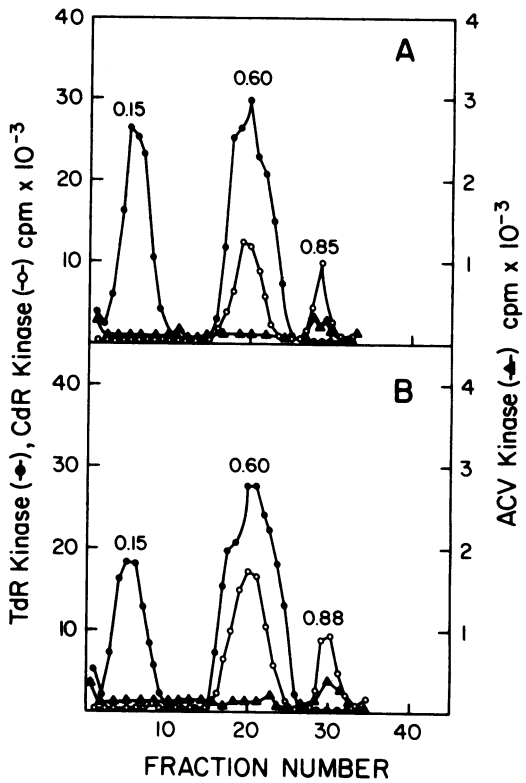

FIG. 3. PAGE analysis of total cellular extracts from IUdR-treated (A) and untreated (B) D98/HR-1 cells for different kinase activities.

these two activities was checked by differential inhibition with dTTP and dCTP and by phosphate-donor specificities (data not presented) (13). Interestingly, we did not detect any other TdR kinase activity peaks in IUdR-treated cells. These results indicated that EBV, unlike HSV, does not code for its own distinct TdR kinase as reported earlier (3).

In addition to cellular TdR-phosphorylating activity, mitochondrial TdR kinase also has CdR kinase activity (14). In addition, there is another activity peak with an electrophoretic mobility of 0.85 to 0.88 that can phosphorylate CdR. This activity has been designated as cytosol CdR kinase (12). In the PAGE analysis, a small fraction (5 to 10%) of the loaded ACV-phosphorylating activity coincided with the cytosol CdR kinase activity in both treated and untreated cells, whereas none of the TdR kinase activities could phosphorylate ACV.

Effect of end product inhibitors on ACV phosphorylation. Reports from several laboratories indicate that dTTP and dCTP are the two key deoxynucleoside triphosphates known to inhibit both cellular and viral TdR kinase activities

strongly (12, 13). However, in our hands dTTP and dCTP each at a concentration of 1 mM inhibited ACV-phosphorylating activity only partially (Table 2). The extent of inhibition was similar in both IUdR-treated and untreated cells.

DISCUSSION

This report substantiates our recent inferences (7, 19) that all herpesviruses do not have to have their own TdR kinase for ACV to be effective. This situation is in contrast to the HSV system in which viral TdR kinase plays an essential role in the phosphorylation of ACV (10). The occasional induction of total cellular TdR kinase activity in superinfected Raji cells (19, 20) is probably dependent on the cell cycle and the stages at which the cells are infected. The results described in this paper with EBV are in agreement with the findings of Burn et al. (2) who showed that mouse cytomegalovirus, which does not code for its own TdR kinase (18), is as sensitive as HSV to ACV.

In view of the reports (1, 12) that cytosol CdR kinase can phosphorylate a large variety of nucleosides other than CdR and that the enzyme has other allosteric sites in addition to the CdR-phosphorylating site, it is not unlikely that cytosol CdR kinase might phosphorylate ACV to some extent. The results (Fig. 3) indicate that a small portion (5 to 10%) of the ACV-phosphorylating activity loaded on the gel seems to migrate with cytosol CdR kinase activity. We could not account for the remaining activity. However, 80 to 85% of TdR kinase activity loaded on the gel could be accounted for. Whether this slight disparity was due to inactivation of the rest of the ACV-phosphorylating activity or to nonmigration of other enzymes into the gel under this electrophoretic condition is not known.

The conclusion that ACV is not phosphorylated by virus-specified TdR kinase in lymphoblastoid cells came from the studies with nucleoside triphosphate inhibition specificity (Table 2). Moreover, phosphorylation of ACV over a wide range of pH (Fig. 2) also supported this conclusion. Partial inhibition of ACV phosphorylation by dTTP and dCTP was probably due to the intricate mechanism of action of these triphosphates toward other nucleoside-phosphorylating activities of the DNA biosynthesis pathway (12).

Our unpublished data on the partial inhibition of ACV-phosphorylating activity by deoxyguanosine, TdR, and CdR are in agreement with the findings of Davidson et al. (9), who showed that cellular toxicity in vivo by ACV can be reversed by the exogenous addition of the above deoxynucleosides. Direct analysis with purified enzymes should throw more light onto this question, but low levels of ACV phosphorylation and the relatively unstable nature of the activity might hinder the execution of such a study.

TABLE 2. Effect of dTTP and dCTP on ACV-phosphorylating activity from cytosol of IUdR-treated and untreated D98/HR-1 cells[a]

Triphosphate	pmol of ACV phosphorylated per mg of protein	
	Untreated	IUdR treated
ATP	923	971
ATP + dTTP (1 mM)	413	419
ATP + dCTP (1 mM)	417	491
None		

[a] Assays were carried out in the presence of 10 mM ATP-Mg^{2+}.

ACKNOWLEDGMENTS

This work was supported by grant I-ROI-AI17205-03VR from the National Institute of Allergy and Infectious Diseases.

We thank B. Leonard and K. Baldwin for secretarial assistance, Y.-C. Cheng for reviewing the manuscript, and the Burroughs Wellcome Co. for the gifts of [^{14}C]ACV and unlabeled ACV.

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